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**Attn: 8(e) Coordinator**

Dear Sir or Madam;

**Contains No CBI**

Attached is a preprint of an article to be published in Environmental Health Perspectives entitled, "Gestational/neonatal exposure of rats to environmental estrogenic chemicals results in reduced testicular size and sperm production in adult life", by Sharpe, et al. (1). This preprint, containing information regarding "4-tert-octylphenol" and a 5-mole ethoxylate of "octylphenol", has come to the attention of Union Carbide and may be considered by the Agency to be reportable under TSCA Section 8(e).

It is in the opinion of Union Carbide that the data presented do not currently represent substantial risk for a variety of scientific reasons. These include: the study design appears inadequate to evaluate the sensitive endpoints discussed; the effects attributed to the test substances are inconsistent with the positive control substance; the magnitude of the changes is inconsistent between studies as well as with the positive control substance; from the types of results (increases and decreases in some measurements), it appears that a multiple comparison statistical method is required but the statistics are not clearly defined and apparently do not take into account the large number of variables; the stated significant differences appear, in many cases, to be suspect based on the standard deviations and range overlap; and, lastly, the article is to be published in a non-refereed journal which prevents thorough peer review to resolve the issues outlined here.

This article and two additional published documents ["Oestrogenic Activity of an Environmentally Persistent Alkylphenol in the Reproductive Tract but not the Brain of Rodents" (2) and "Effects of Trace Organics on Fish - Phase 2" (Executive Summary

only) (3)], are being submitted by Union Carbide for your information. The two additional articles do not, to our knowledge, contain new information suggestive of substantial risk or effects reliably ascribed to chemicals produced or processed by Union Carbide.

Please contact the undersigned with questions, if any, at 203/794-5230.

Very truly yours,



William C. Kuryla, Ph.D.  
Associate Director  
Product Safety

WCK/jr

Attachments

- (1) "Gestational/neonatal exposure of rats to environmental estrogenic chemicals results in reduced testicular size and sperm production in adult life", R. M. Sharpe, J. S. Fisher, M. M. Millar, S. Jobling, J. P. Sumpter; Environmental Health Perspectives (In Press).
- (2) "Oestrogenic Activity of an Environmentally Persistent Alkylphenol in the Reproductive Tract but not the Brain of Rodents", R. J. Bicknell, A. E. Herbison, J. P. Sumpter; J. Steroid Biochem. Molec. Biol., Vol. 54 (1/2), pp. 7-9, 1995.
- (3) "Effects of Trace Organics on Fish", J. E. Harries, S. Jobling, P. Matthiewssen, D. A. Sheahan, J. P. Sumpter; Contents and Executive Summary, Prepared for the Dept. of the Environment (U.K.), FR/D 0022, July, 1995.

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Environ Health Perspect. (In Press)

**Gestational/ neonatal exposure of rats  
to environmental estrogenic chemicals results  
in reduced testicular size and sperm production in adult life**

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## Abstract

This study has assessed whether exposure of male rats to two estrogenic, environmental chemicals, octylphenol (OP) or butyl benzyl phthalate (BBP) during gestation and/or the first 21 days of postnatal life, affected testicular size or spermatogenesis in adulthood (90-95 days of age). Chemicals were administered via the drinking water at concentrations of 10-1000  $\mu\text{g/L}$  (OP) or 1000  $\mu\text{g/L}$  (BBP); diethylstilbestrol (DES) (100  $\mu\text{g/L}$ ) and an octylphenol polyethoxylate which is very weakly or non-estrogenic in-vitro (OP-5EO; 1000  $\mu\text{g/L}$ ) were administered as presumptive positive and negative controls, respectively. Overall controls received the vehicle (ethanol) in tapwater. In Study 1, rats were treated from days 1-22 postnatal, whereas in Studies 2 and 3, the adult female mothers were exposed to treatment for approximately 8-9 weeks, spanning a 2-week period prior to mating, throughout gestation and up until day 22 postnatal.

With the exception of DES, treatment generally had no major adverse effect on bodyweight; indeed, in most instances, treated animals were heavier than controls at day 22 and at day 90-95. Exposure to OP, OP-5EO or BBP at a concentration of 1000  $\mu\text{g/L}$  resulted in a small (5-13 %) but significant ( $p < 0.01$  or  $p < 0.001$ ) reduction in mean testicular size in both Studies 2 and 3, an effect that was still evident when testicular weight was expressed relative to bodyweight or kidney weight; the effect of OP-5EO is attributed to its metabolism in-vivo to OP. DES-exposure caused similar reductions in testicular size but also caused reductions in bodyweight, kidney weight and litter size. Ventral prostate weight was reduced significantly in DES-treated rats and to a minor extent in OP-treated rats. Comparable, but more minor, effects of treatment with DES or OP on testicular size were observed in Study 1. None of the treatments had any adverse effect on testicular morphology or on the cross-sectional area of the lumen or seminiferous epithelium at stages VII-VIII of the spermatogenic cycle, but DES, OP and BBP caused reductions of 10-21% ( $p < 0.05$  to  $p < 0.001$ ) in daily sperm production. Humans are exposed to phthalates, such as BBP, and to alkylphenol polyethoxylates, such as OP, though the level of exposure is poorly described. The present data suggest that more detailed studies are warranted to

assess the possible risk to development of the human testis from exposure to these and other environmental estrogens.

**Keywords:** Sertoli cell number, spermatogenesis, daily sperm production, 4-octylphenol, butyl benzyl phthalate, diethylstilbestrol

## Introduction

The report by Carlsen *et al.* (1) that mean sperm counts in unselected men had apparently declined by around 40-50% over the past half-century, was greeted with a mixture of concern (e.g. ref 2) and scepticism (e.g. ref 3). The most recent data from a number of countries, which have charted changes in sperm counts in semen donors over the past 20-25 years, have, however, all reported a marked and significant downward trend(4-6). The most comprehensive of these studies, in Paris, concluded that sperm counts in *fertile* men have declined by around 2% per year over the past 23 years (6). Moreover, two of the cited studies (5,6) identified that the temporal decline in sperm counts appears to apply to men born from around 1950 onwards.

Two years ago, we hypothesized (7) that the reported decline in sperm counts might be related to an increasing incidence of other disorders of development of the male reproductive system (e.g. testicular cancer), and that this could have arisen because of increased exposure of the developing fetus/neonate to estrogens. One potential source of this increased estrogen exposure was via 'environmental estrogenic chemicals', the release of which into the environment has more or less coincided with the decline in sperm counts (8,9). Concern about such hormonally active pollutants (e.g. chlorinated pesticides) has been voiced for 10-20 years (10), but has become more acute recently because of the discovery of a range of new estrogenic environmental chemicals, including bisphenol-A (11), certain alkylphenolic chemicals (12-14), certain phthalates (15) as well as a number of pesticides (16). Most of these estrogenic chemicals are ubiquitous in the environment and humans are exposed to them daily by a number of routes (9). However, the risk to man from these chemicals is presently theoretical because there is no data to show that these chemicals are capable of causing any disorder of reproductive development or function in animals.

Pathways via which exposure of the developing male fetus/neonate to estrogenic chemicals could result in reduced testicular size and sperm output in adult life have been identified (2,7,9), but there is no direct evidence to confirm whether this hypothesis has any factual basis. However, it is known that some phthalates are passed from the mother both

across the placenta (17) and via milk (18) to the developing young, although comparable data on the transfer of alkylphenolic chemicals is lacking. The aim of the present studies was to evaluate in a preliminary way whether exposure of the male rat fetus/neonate to either of two environmental estrogenic chemicals had any effect on testicular size and spermatogenesis in adult life.

## **Material and Methods**

### ***Animals and treatments***

All rats used in these studies were of the Wistar strain and were bred in our own animal facility. They were maintained under standard, controlled conditions and had free access to food and water. Administration of chemicals was via the drinking water which was provided in a bottle per cage. A stock solution of each dose of chemicals was made by dissolving a weighed amount in ethanol such that addition of 0.5 ml of this stock to 5 litres of tapwater resulted in the test dose; control animals had 0.5 ml ethanol/5 L added to their drinking water.

### ***Study design***

The most likely mechanism via which estrogens/estrogenic chemicals could cause an irreversible reduction in testicular size and sperm output is by decreasing the number of Sertoli cells as, in adult life, the number of these cells determines testicular size and sperm output in all animals that have been studied (reviewed in ref 19). In the male rat, multiplication of Sertoli cells commences soon after testicular differentiation (c. day 15 of gestation) and continues until around day 15 of postnatal life, with perhaps minor multiplication until around day 21; after this time, no further Sertoli cell multiplication can occur (19). Thus, by day 22, the ultimate size to which the testis will grow in adulthood (90-95 days of age) has been predetermined (19-22).

The present studies were designed such that animals were exposed to chemicals for either the postnatal period (i.e. days 1-22 postnatal) of Sertoli cell multiplication (Study 1; see Fig. 1) or for the complete period of Sertoli cell multiplication (Studies 2 & 3; see Fig. 1). In the latter two studies, treatments were administered to adult female rats for 2 weeks

prior to mating with a sexually-experienced male, throughout mating, throughout gestation and up until day 22 postnatal (Fig. 1). This protocol of exposure was used to assess the possible effects of bio-accumulation. In all 3 studies, exposure of the male offspring to the test chemicals was thus largely indirect i.e. via the placenta and/or milk.

In Study 1, in which there was no prenatal exposure to the test chemicals, litters were culled to 8 pups on the day of birth (=day 1) by culling excess females. The same was done in Study 2, whereas in Study 3, the full natural litter size was maintained from birth through to day 22. The female offspring of test litters were not evaluated. It should also be noted that the adult females used for mating in Studies 2 and 3 were the same i.e. when offspring of these females were weaned at the completion of Study 2, the mothers were maintained on the same treatment/dose for two weeks, then mated and exposure continued until the weaning of Study 3 offspring (Fig. 1).

#### *Test chemicals and doses*

Three chemicals were selected for study based on the results of in-vitro investigations suggesting that they were estrogenic (13-15). Of the three, OP (4-tert-octylphenol; Aldrich Chemical Co., Gillingham, UK) and Butyl benzyl phthalate (BBP; Chem Service, West Chester, PA ) were both demonstrated to be estrogenic in-vitro, whereas an octylphenol polyethoxylate which had a side-chain of 5 ethoxylate groups (OP-5EO; Igepal CO-520; Aldrich Chemical Co. ) was shown to be essentially devoid of estrogenicity in-vitro. In Study 1 only, nonylphenoxycarboxylic acid (NP1EC, K & K Labs, ICN, Cleveland, Ohio), which is approximately 10-fold less estrogenic in-vitro than OP (14), was also assessed at a single dose. Diethylstilboestrol (DES; Sigma Chemical Co., Poole, Dorset, UK), which is a potent non-steroidal estrogen, was included as a positive control.

Relatively little is known about the degree of exposure of humans to the chemicals used in the present studies, but concentrations of alkylphenolic compounds in the aquatic environment reportedly range up to hundreds of  $\mu\text{g}/\text{ml}$  (23,24) whereas human intake of phthalates is reportedly as high as 15 mg per day (=200-300  $\mu\text{g}/\text{kg}/\text{day}$ ) (ref 25). Therefore, the aim was to use doses which would be mildly estrogenic based on in vitro

analyses (14,15) but which remained within an order of magnitude of the possible environmental/human intake level. OP was tested at 1000 and 100 µg/L in all three Studies (and also at 10 µg/L in Study 1), whereas OP-SEO and BBP were tested only at the single dose of 1000 µg/L in Studies 2 and 3. DES was tested at 100 and 10 µg/L in Study 1 and at 100 µg/L in Studies 2 and 3. No formal confirmation that the test chemicals (or their metabolites) actually reached the male offspring, or in what amounts, was obtained in the present studies, as the objective was simply to establish whether or not the chemicals exerted any biological effects. However, water intake, and thus the nominal intake of chemical/day, was assessed in some of the treatment groups in Study 3, by weighing water bottles every 48 hours.

#### *Body and organ weights, litter size and composition*

In Studies 2 and 3, litter size and composition at birth was recorded. In all three Studies, the male offspring were weighed at weaning (=day 22), which was the day that treatment ceased. The male offspring were then maintained in their litters under standard animal house conditions until 90-95 days of age, when they were killed by inhalation of CO<sub>2</sub>, followed by cervical dislocation. Bodyweight was recorded and the right testis, left kidney and ventral prostate were dissected out and weighed; the epididymis, seminal vesicles and left testis were also inspected macroscopically for any obvious abnormalities. Kidney weight was recorded because it is an organ which varies according to bodyweight but was not expected to be affected by the experimental manipulations. It was thus used as an internal 'organ control' for the specificity of any effects observed on testis size.

#### *Testicular morphology*

At 90-95 days of age, representative animals from the various treatment groups were fixed with 3% glutaraldehyde in 0.2 M Cacodylate buffer by perfusion via the dorsal aorta, as described elsewhere (26). The fixed testes were then cut transversely with a razor blade into 2 mm thick sections and then into small blocks (1-2 mm<sup>2</sup>). After postfixation for 12-16 h in the same fixative, the blocks were processed and embedded in plastic as described

previously (26). Semi-thin sections (0.5  $\mu$ m) were then cut, stained with Toluidine blue and examined using a Zeiss photomicroscope.

To provide a preliminary quantitative assessment of the normality of spermatogenesis, seminiferous tubules at stages VII-VIII of the spermatogenic cycle were subjected to image analysis (Cue-2, Olympus) to determine the cross-sectional area of the seminiferous tubule and seminiferous epithelium (27). Ten round cross-sections were analysed for 2 or 3 rats per treatment group and the mean  $\pm$  SD for each animal then calculated. Stages VII-VIII were chosen for analysis as they contain representative germ cells from all steps of development (19).

*Daily sperm production (DSP)*

In some of the animals from Study 3, DSP was determined by the enumeration of homogenization-resistant spermatids, using minor modifications of the techniques of Johnson *et al* (28,29). A 500-700 mg portion of testicular tissue recovered at autopsy was immersion fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer and kept at 4°C until used for DSP determination within the next 6 weeks. The tissue was then removed, blotted and weighed and two ~50 mg portions cut with a scalpel, weighed and then homogenized separately in 5 ml 0.15 M NaCl, 0.05% Triton-X100, 0.025% sodium azide using a Kinematica Polytron (PT-K/PCU-8) homogenizer at speed 5 for 60 secs (these conditions had previously been validated and optimized). Using a haemocytometer, homogenization-resistant step 18 and 19 spermatids were then counted separately in 3 aliquots of each of the two homogenates per sample and the mean of the six measurements calculated; the coefficient of variation for these replicates averaged 7% for all samples. This value was then corrected for sample weight and overall testis weight, and transformed to the DSP by dividing by the appropriate time divisor (=4.61) based on the proportional duration of stages VI-VIII in days according to Leblond & Clermont (30). Confirmation that only step 18 and 19 spermatids were being counted was provided by application of the above procedures to known lengths of seminiferous tubule isolated from normal adult rats by

transillumination-assisted microdissection (31) at stages II-V (containing step 16-17 spermatids) and VI-VIII (containing step 18 and 19 spermatids).

#### *Statistical analysis*

In each of the 3 Studies, each parameter in the different treatment groups was subjected to analysis of variance to determine whether there were significant effects of treatment. Where these were indicated, sub-group comparisons between means for the control and each treatment group were made using the variance from the Study as a whole as the measure of error. All data were normally distributed, so no transformations were made and results are all reported as Means  $\pm$  SD.

#### *Results*

##### *Litter size and composition and bodyweight at weaning*

Litter size and composition at birth were not evaluated in Study 1 as there had been no prenatal treatment of the mothers. In Studies 2 and 3, in which the mothers were treated prenatally, there was no effect of treatment with OP, OP-5EO or BBP on litter size or composition but exposure to DES (100  $\mu$ g/L) reduced average litter size by nearly half in Study 3 and had a more minor effect in Study 2 (Table 1). Curiously, the proportion of male offspring was increased significantly in the DES-exposed group in Study 3 (Table 1).

At weaning, which corresponded to the cessation of treatment in all 3 studies, bodyweight of DES-exposed offspring was reduced significantly in Studies 1 and 2 but was increased significantly in Study 3, perhaps because of the much smaller litter sizes (Table 1). Otherwise, exposure to any of the test chemicals had either no effect or, more commonly, resulted in a significant increase in bodyweight on day 22 (Table 1).

##### *Body and organ weights in adult life*

*Study 1:* Mean bodyweight was generally higher in treatment groups, compared with controls, but only in the case of OP (100  $\mu$ g/L) did this reach statistical significance (Table

2). Average testis weight was reduced marginally, but significantly, in DES (100 µg/L) and OP (1000 µg/L) -exposed animals and relative testis weight (i.e. relative to bodyweight or to kidney weight) was reduced significantly in these two groups and in animals exposed to the intermediate concentration (100 µg/L) of OP (Table 2). Kidney weight was increased quite markedly in animals exposed to the top two doses of OP and, although this may have been due to some extent to the greater average bodyweight of the animals, a significant difference in kidney weight relative to bodyweight was still evident (Table 2).

*Study 2:* Mean bodyweight in animals exposed to DES or OP-5EO was reduced when compared to controls, whereas animals exposed to either dose of OP were increased in size by 8% or more (Table 3). Except for animals exposed to the lower dose of OP (100 µg/L), all other treatment groups exhibited a highly significant decrease in absolute testis weight and in the ratio of testis/kidney size (Table 3); all treatment groups showed a significant decrease in relative testis weight. There were some minor, but significant, changes in absolute and relative kidney weight in some of the treatment groups. Ventral prostate weight was reduced by 16% in DES-treated animals, though this effect largely disappeared when the size relative to bodyweight was evaluated (Table 3). Relative weight of the prostate was reduced significantly in animals exposed to either dose of OP.

*Study 3:* In comparison to the results obtained in Studies 1 and 2, animals reared to weaning in their natural litter sizes were noticeably smaller on average, both at weaning and at 90-95 days of age (Table 4). Under this regimen, no treatment group in adult life had a larger mean bodyweight than the control group and two of the groups (OP 100 µg/L and OP-5EO) showed a small but significant decrease in bodyweight relative to controls. In all treatment groups, testis weight was reduced significantly in size when compared to controls, though when expressed relative to bodyweight this difference disappeared for the groups exposed to DES or the lower dose of OP (Table 4). Kidney weight was reduced noticeably in size in animals exposed to 1000 µg OP/L, a difference still evident when expressed relative to bodyweight; however, the testis/kidney weight ratio in this group

was still significantly lower than that observed in the control group (Table 4). As in Study 2, ventral prostate weight was reduced significantly in DES-exposed animals but, in this Study, a significant reduction was also obvious in animals exposed to OP, particularly at the higher dose (Table 4).

#### *Testis size in Studies 2+3*

Although exposure to the test chemicals throughout gestation/neonatal life resulted in fairly consistent reductions in testis size in adult life, these decreases were only of the order of 5-13%. However, plotting of data for testis weight against bodyweight for four of the treatment groups from Studies 2+3 (i.e. DES, OP 1000 µg/L, OP-5EO, BBP) provides evidence that the treated animals come from a different population to the controls (Fig. 2). This is most evident by noting how few of the values for treated animals lie above the linear regression line plotted for the control group. Although a similar trend was evident in the DES-exposed animals, testicular weights were far more variable in this treatment group, probably because of the confounding effects of this treatment on litter size etc.

#### *Testicular morphology and daily sperm production (DSP)*

Testicular morphology was indistinguishable in animals from the control and treatment groups and no obvious abnormalities in the seminiferous tubules, interstitium or vasculature were evident (Fig. 3). Image analysis confirmed this impression by demonstrating no adverse effect of treatment on the cross-sectional parameters of stage VII/VIII seminiferous tubules; indeed, for the most part these parameters tended to be higher for the treated animals than for the controls, though this is based on only a small sample size (Table 5).

DSP in control animals from Study 3 averaged  $24.9 \pm 3.6 \times 10^6$  per testis per day (Mean  $\pm$  SD, n=12), which agrees closely with that determined morphometrically by Wing & Christensen (32). Animals exposed during fetal/neonatal life to DES, OP (1000 µg/L) or BBP in Study 3, all showed significant reductions of 10-21% in the mean DSP (Fig. 4) which

were proportionately similar to the decrease in testis weight (Table 4); tissue from animals exposed to OP-5EO was not evaluated.

#### *Level of chemical exposure*

The nominal intake of chemical was assessed in Study 3 for animals in two of the treatment groups (OP 1000  $\mu\text{g}/\text{L}$ , BRP) based on water intake, and ranged from around 125  $\mu\text{g}/\text{kg}/\text{day}$  in the first two days after birth to 370  $\mu\text{g}/\text{kg}/\text{day}$  just before weaning (Table 6). As these calculations take no account of spillage, adsorption or degradation of the chemicals (which were not evaluated), these values for intake can be viewed as over-estimates of the actual intake. The level of water intake was not affected by treatment (Table 6).

#### **Discussion**

The purpose of the present studies was to assess whether exposure of male rats to known estrogenic, environmental chemicals during gestation and/or neonatal life had any adverse effect on testicular size and spermatogenesis when these animals reached adulthood. The results are unequivocal in showing that exposure to such chemicals does cause a reproducible and consistent decrease in ultimate testicular size and daily sperm production (DSP) in rats, an effect which cannot be attributed to any obvious overt toxicity (judged on bodyweight and kidney weight). Although the chemical-induced decrease in testicular size and DSP only ranged from 5-21%, this effect occurred during a relatively short period of treatment and after exposure to relatively low levels of the various chemicals. Previous data involving a similar protocol of exposure of rats to the estrogenic pesticide, methoxychlor, also reported a small reduction in adult testicular size and sperm counts (34), and a recent study in trout (35) has demonstrated inhibition of testis growth *in vivo* after exposure to estrogenic alkylphenolic chemicals.

Ultimate testicular size in all mammals that have been investigated is determined by the number of Sertoli cells present in the testis, despite the fact that it is the germ cells, rather than the Sertoli cells, which constitute the bulk of the testis (19,21). This is because

each Sertoli cell can only support a fixed number of germ cells through their development into spermatozoa, and hence the more Sertoli cells that are present the more germ cells will be present, and thus the larger the testis. The number of Sertoli cells can be manipulated experimentally up or down, in various animals, by a number of treatments, with corresponding changes in testicular size and DSP, and the same relationship appears to apply to man (19). Usually, when Sertoli cell number is altered, there is little change in the cross-sectional appearance or size of the seminiferous tubules because Sertoli cell number affects primarily the length, not the breadth, of the tubules (20,21). The number of Sertoli cells per testis is determined by the rate and duration of their multiplication, events which occur usually during a precisely timed period which commences in fetal life (shortly after testicular differentiation) and which continues out into neonatal/postnatal life for a period which varies according to the species (19). In the present studies, rats were exposed to estrogenic chemicals for either part (Study 1) or all (Studies 2 and 3) of the period when Sertoli cell multiplication occurs (Fig. 1). If these treatments had reduced the rate of Sertoli cell multiplication, the expectation would be that, in adult life, the testes would be smaller and DSP reduced but the cross-sectional appearance of the seminiferous tubules would probably be unchanged. As this was what was observed, the findings are consistent with the treatments having reduced Sertoli cell number, though morphometric determination of Sertoli cell number will be necessary to determine whether this interpretation is correct; as the expected change in Sertoli cell number would only be of the order of 2-4%, measurements in large cohorts of animals would be necessary to demonstrate this. However, it is worth noting that an earlier study (33) demonstrated that exposure of rats to di (2-ethylhexyl) phthalate for 5 days during neonatal life, resulted in a reduction in Sertoli cell number and some reduction in testis size and sperm production in adulthood; however, the level of phthalate exposure in this study was at least 1000-fold higher than in the present studies.

When animals are exposed to test chemicals, it is possible that toxic effects on organs (e.g. the liver) other than the testis or reproductive axis could lead, secondarily, to a reduction in testicular size and thus DSP as a result of 'non-specific' effects. Whilst this

possibility cannot be excluded with complete confidence, the present data on bodyweight and kidney weight provides little evidence for any such effect - indeed, in most instances treated animals were larger than were the controls. The exception was animals exposed to DES (=positive controls) which showed consistent evidence of somewhat lower bodyweights. The explanation for this effect is not clear from the present studies, but it is likely that lactation may have been impaired by DES (34). If this is the case, it is somewhat puzzling why no evidence for such effects was evident in animals exposed to any of the estrogenic chemicals, as these caused equal, or even larger, reductions in testicular weight than did DES exposure. This discrepancy could reflect differences in the pharmacokinetics of the chemicals compared with DES.

Although the estrogenic chemicals tested in the present studies exerted similar effects on testis size and DSP, no evidence is provided that these effects resulted *specifically* from the estrogenicity of these compounds. Indeed, the fact that treatment with OP-5EO caused a similar reduction in testicular size as did treatment with OP, despite the fact that the former is non-estrogenic *in-vitro* (14), could be interpreted as evidence against estrogenicity *per se* being a common causal mechanism. However, it is possible that, when ingested, OP-5EO is metabolized such that the 5EO are cleaved, resulting in the formation of OP. This interpretation is supported by the observation that whereas short-chain alkylphenol polyethoxylates (such as OP-5EO) do not bind to the estrogen receptor in cell-free systems (14), they are estrogenic in cell-based *in-vitro* assays (13,14) and *in-vivo* (35). This would seem to offer a reasonable explanation for the present observations, although it will be important in future studies to establish, unequivocally, whether only estrogenic chemicals are able to reduce testicular size and DSP in the manner described in the present investigation.

Irrespective of whether the reduction in testis size and DSP caused by developmental exposure to OP or BBP resulted from their estrogenicity, the key question which their effects raise is whether it has relevance to man. This is a complex issue which requires detailed dose-response data and measurement of the actual levels of the administered chemicals in the male rats during fetal/neonatal life. These are beyond the

scope of the present study. However, it would seem appropriate to consider briefly whether the nominal level of exposure of rats to OP and BBP in the present studies (which is presumed to be an over-estimate of actual exposure levels) bears any relationship to the equivalent level of human exposure. There is little or no data specifically for OP, but there is some information on the environmental levels of the class of compounds to which OP belongs, namely alkylphenol polyethoxylates, several of which have been shown to be estrogenic (14). Reported levels of these compounds in river water vary from the low  $\mu\text{g/L}$  (44) to tens and hundreds of  $\mu\text{g/L}$  (23,24), which approach the nominal levels of exposure in the present study. Even tapwater has been reported to contain estrogenic degradation products of both nonylphenol-EO and octylphenol-EO (36), although the combined concentration was only about 1  $\mu\text{g/L}$ . However, as APs are used widely in industrial and some household detergents/cleaners, in certain plastics and in many other ways, human exposure via routes other than drinking water are likely.

In the case of BBP, there is perhaps more evidence for concern about the possible risk to human health. BBP and other phthalates are the most ubiquitous of all environmental contaminants, primarily because of their use as plasticizers, and human exposure is likely to be high (25,37,38). For example, a recent study reported levels of BBP alone, as high as 47.8 mg/kg in some foil-wrapped butters (39), which would mean that ingestion of 50 g/day of such butter by a 60 kg woman would equate to an intake of approximately 40  $\mu\text{g/kg/day}$ , which approaches the nominal intake values in the present study. As the levels of total phthalates in other dairy produce can exceed the figure quoted above (40,41), and there are many other possible sources of human exposure to these compounds, the present findings suggest that further studies of the estrogenicity of phthalates should have some degree of priority. There is already a huge literature on the toxicity of phthalates, including their testicular toxicity, but few of the published studies have had an experimental design which would detect possible developmental effects similar to those reported here. This is borne out by the reported no-effect level of BBP for testicular toxicity of 125-150 mg/kg/day (42,43). The fact that, in the present studies, nominal intake of 300-fold lower amounts than this, resulted in around a 10% decrease in

testicular weight in two separate studies with a commensurate fall in DSP, argues further that the cause of these decreases differs from the previously reported toxic effects of these compounds on the testis.

The present data do not provide any direct evidence which enables a link between human exposure to environmental estrogens and falling sperm counts in man to be established. However, the findings do provide some preliminary, indirect evidence that exposure of rats to certain environmental estrogenic chemicals during gestation and/or neonatal life can result in reduced testicular size and sperm output in adulthood. As these effects occurred in rats after only 3-9 weeks of exposure, whereas in man the corresponding window of development (and Sertoli cell multiplication) spans several years, there is at least the theoretical possibility that similar effects in man might be of larger magnitude than those described here for the rat. However, considerably more work, particularly in establishing the likely level of human exposure to estrogenic chemicals, will be necessary if the risk to man from such exposure is to be assessed with any accuracy.

#### Acknowledgements

We are grateful to Jim McDonald for expert help with the animals in these studies.

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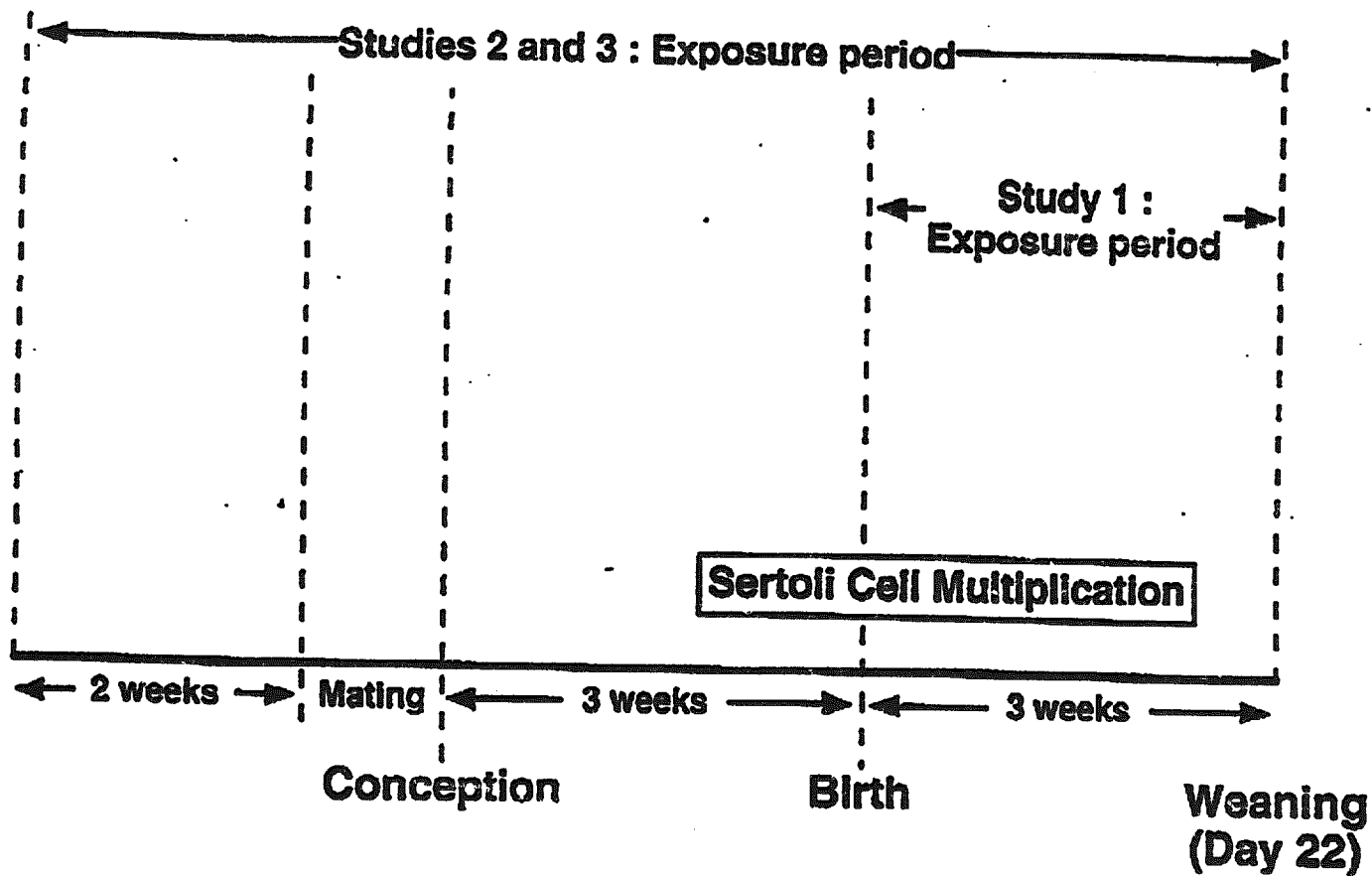
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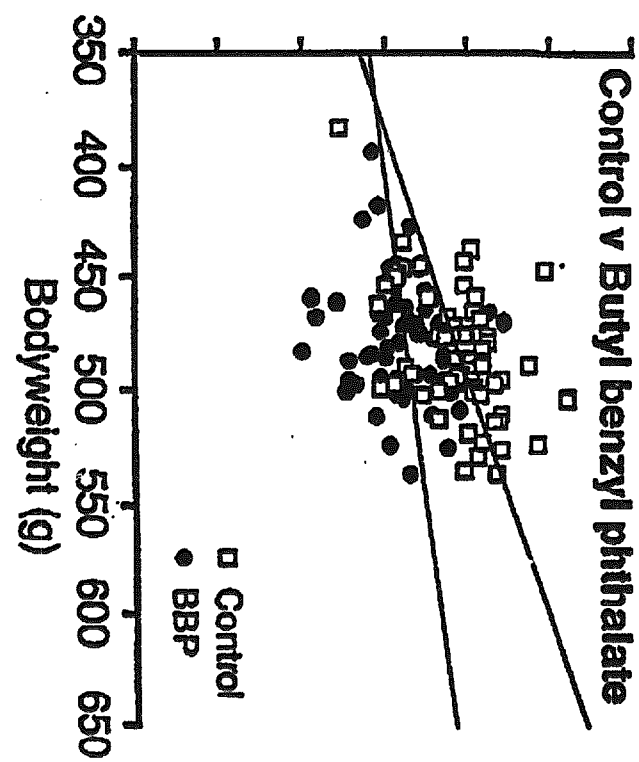
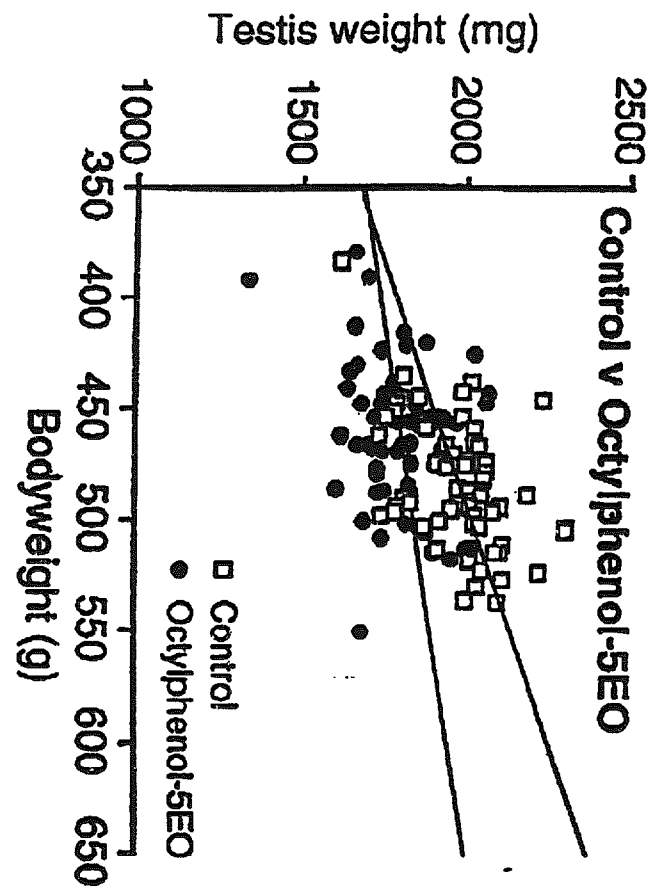
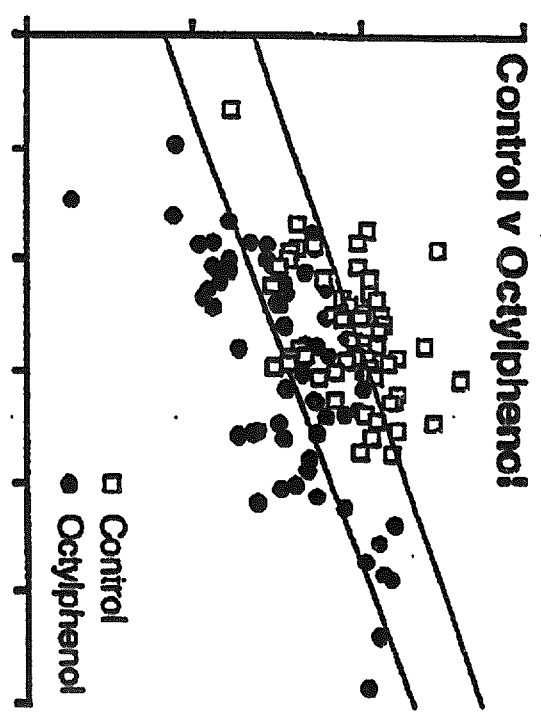
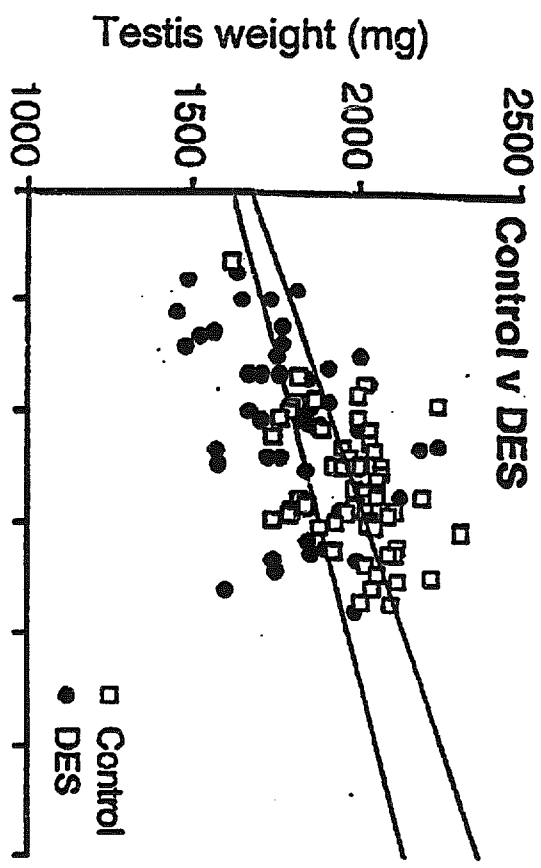
**Figure 1.** Experimental design of the present studies, indicating the periods of treatment in relation to the time of normal multiplication of Sertoli cells. Following cessation of treatment (day 22 postnatal) animals were maintained under normal conditions until they were killed at the age of 90-95 days.

**Figure 2.** Scatter plots of testicular size versus bodyweight for animals from Studies 2 and 3 combined. The data for controls is shown in each of the panels in comparison to that for animals exposed to DES (top left), 1000  $\mu\text{g}$  OP/L (top right), OP-5EO (bottom left) or butyl benzyl phthalate (bottom right). Linear regression lines for the control and each treatment group are shown to aid comparison. Mean values for Studies 2 and 3 are given separately in Tables 3 and 4.

**Figure 3.** Representative testicular morphology in a control animal (top left) and in rats exposed during fetal/neonatal life to DES (top right), 1000  $\mu\text{g}$  OP/L (bottom left) or butyl benzyl phthalate (bottom right) in Study 3. All  $\times 120$  magnification.

**Figure 4.** Daily sperm production (means  $\pm$  SD) in representative control animals ( $n=12$ ) and in rats exposed during fetal/neonatal life to DES ( $n=7$ ), OP (1000  $\mu\text{g}$ /L;  $n=18$ ) or BBP ( $n=7$ ) in Study 3. \*  $p<0.05$ , \*\* $p<0.01$ , \*\*\* $p<0.001$ , in comparison with control.





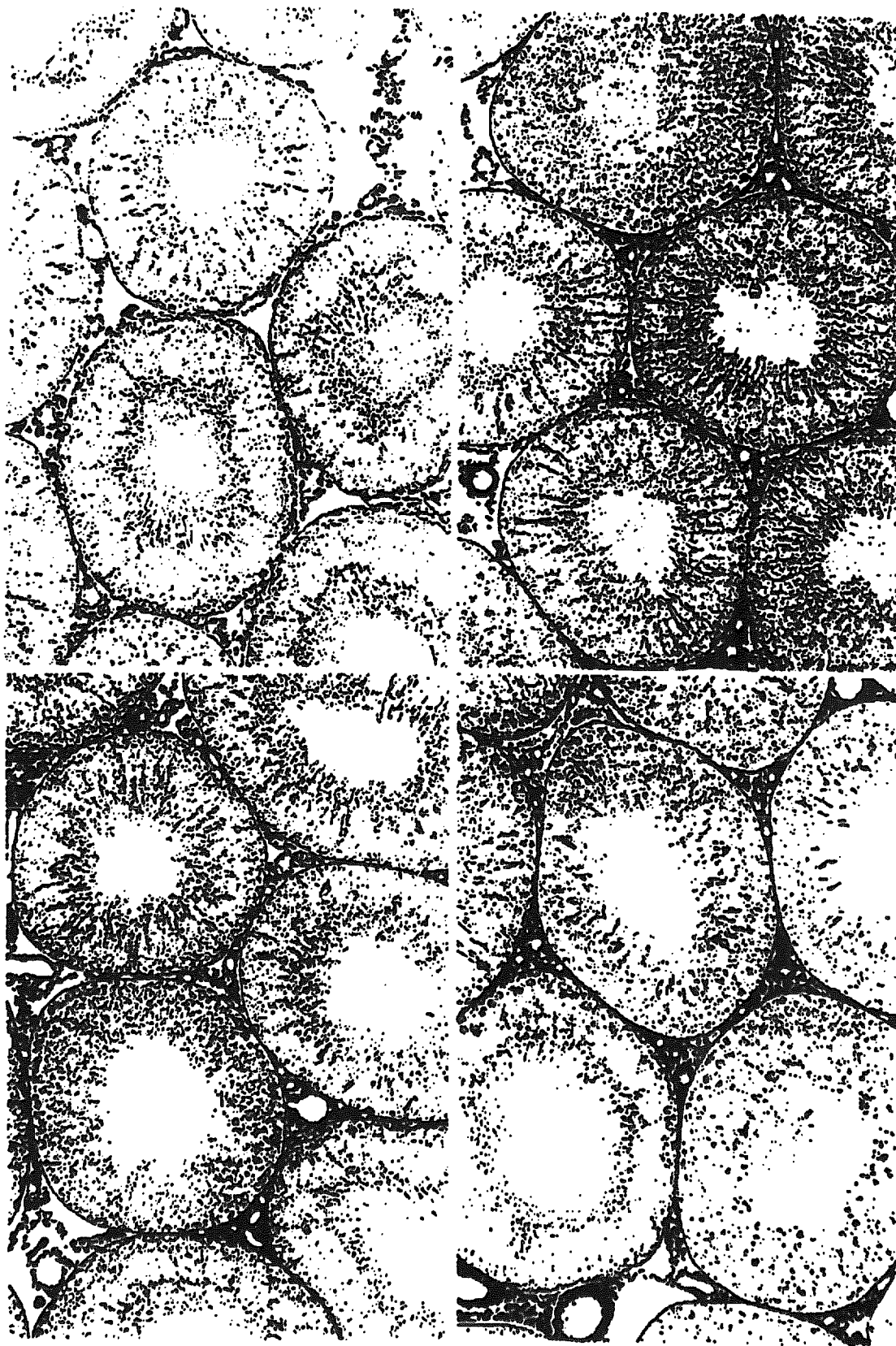
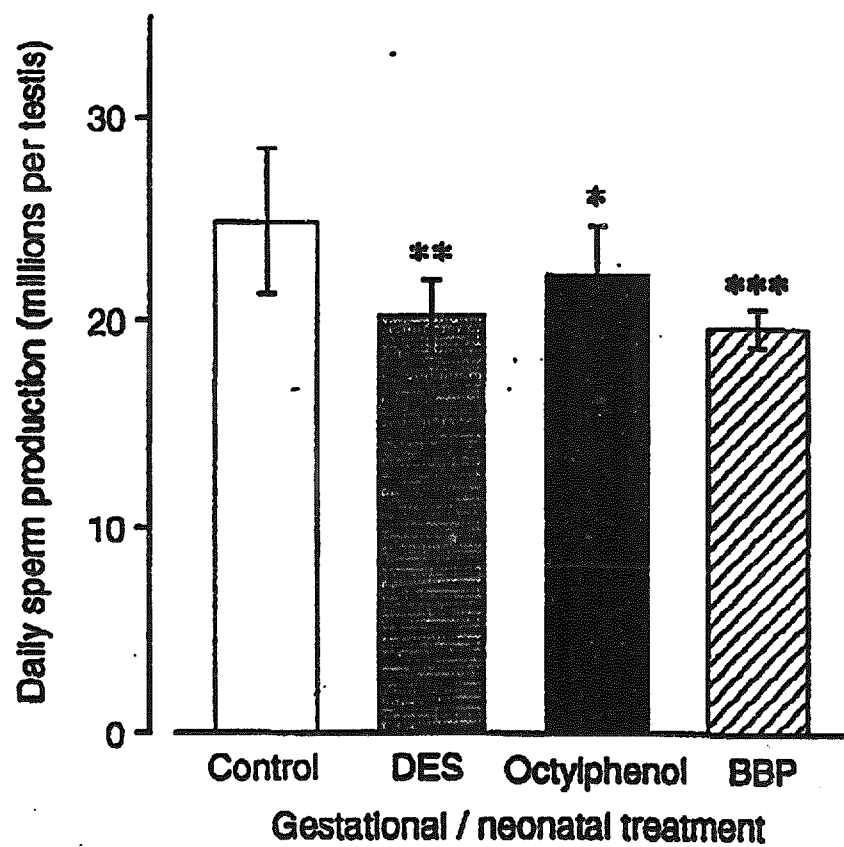


FIG. 4.



**Table 1. Litter size and composition at birth and bodyweight on day 22 in Studies 1, 2 and 3 (Means  $\pm$  SD).**

Study No	Treatment Group ( $\mu\text{g/L}$ )	Litter size	% males at birth	Bodyweight (g) of males on day 22
1	Control	nd	nd	60 $\pm$ 8 (N=70)
	DES - 100	nd	nd	55 $\pm$ 7** (N=46)
	DES - 10	nd	nd	54 $\pm$ 5*** (N=48)
	Octylphenol 1000	nd	nd	60 $\pm$ 5 (N=56)
	Octylphenol - 100	nd	nd	65 $\pm$ 6*** (N=35)
	Octylphenol - 10	nd	nd	65 $\pm$ 9** (N=34)
	Nonylphenol - 100	nd	nd	nd
2	Control	10.0 $\pm$ 2.5 (5) <sup>a</sup>	61 $\pm$ 8	53 $\pm$ 5 (N=29)
	DES 100	8.2 $\pm$ 1.7 (6)	64 $\pm$ 14	46 $\pm$ 8*** (N=30)
	Octylphenol - 1000	12.0 $\pm$ 1.1 (6)	46 $\pm$ 14	61 $\pm$ 8*** (N=30)
	Octylphenol - 100	10.8 $\pm$ 3.5 (6)	53 $\pm$ 14	66 $\pm$ 5*** (N=33)
	Octylphenol - 5EO-1000	10.6 $\pm$ 3.2 (5)	63 $\pm$ 15	54 $\pm$ 7 (N=39)
	BBP - 1000	11.6 $\pm$ 3.4 (5)	64 $\pm$ 13	59 $\pm$ 7** (N=38)
3	Control	12.7 $\pm$ 2.2 (6)	55 $\pm$ 12	50 $\pm$ 10 (N=36)
	DES - 100	6.2 $\pm$ 3.3 (5)**	80 $\pm$ 20*	57 $\pm$ 8** (N=27)
	Octylphenol - 1000	11.2 $\pm$ 1.8 (5)	64 $\pm$ 7	52 $\pm$ 8 (N=39)
	Octylphenol - 100	10.8 $\pm$ 2.1 (6)	57 $\pm$ 13	54 $\pm$ 7* (N=36)
	Octylphenol - 5EO-1000	13.8 $\pm$ 0.4 (6)	45 $\pm$ 11	47 $\pm$ 4 (N=34)
	BBP - 1000	13.4 $\pm$ 1.5 (5)	57 $\pm$ 3	57 $\pm$ 8** (N=38)

\*p<0.05, \*\* p<0.01, \*\*\* p<0.001, in comparison with respective control value

<sup>a</sup> Number of litters

nd-not determined

**Table 2** Effect of exposure of male rats, from birth to day 22, to diethylstilboestrol (DES), octylphenol (OP) or nonylphenoxycetic acid (NP1EC) added to the drinking water ( $\mu\text{g/L}$ ) on bodyweight, testis and kidney weight (Means  $\pm$  SD) at age 90-95 days (Study 1)

Treatment Group ( $\mu\text{g/L}$ ) <sup>a</sup>	Body weight (g)	Testis weight (mg)	Kidney weight (mg)	Testis/Kidney weight ratio	Relative organ weight (mg/g BW)	
					Testis	Kidney
Control (N=65)	504 $\pm$ 66	1968 $\pm$ 163	1739 $\pm$ 172	1.14 $\pm$ 0.14	3.85 $\pm$ 0.38	3.39 $\pm$ 0.32
DES-100 (N=36)	516 $\pm$ 33	1894 $\pm$ 218*	1797 $\pm$ 124	1.06 $\pm$ 0.16**	3.69 $\pm$ 0.49*	3.49 $\pm$ 0.20
DES-10 (N=44)	506 $\pm$ 40	1961 $\pm$ 147	1732 $\pm$ 131	1.16 $\pm$ 0.09	3.89 $\pm$ 0.34	3.37 $\pm$ 0.24
OP-1000 (N=49)	518 $\pm$ 34	1898 $\pm$ 130*	1883 $\pm$ 223***	1.02 $\pm$ 0.12***	3.68 $\pm$ 0.23**	3.64 $\pm$ 0.36**
OP-100 (N=29)	556 $\pm$ 37***	1990 $\pm$ 126	1968 $\pm$ 165***	1.02 $\pm$ 0.10***	3.60 $\pm$ 0.34**	3.54 $\pm$ 0.23*
OP-10 (N=27)	511 $\pm$ 39	1940 $\pm$ 132	1776 $\pm$ 164	1.10 $\pm$ 0.11	3.82 $\pm$ 0.38	3.48 $\pm$ 0.27
NP1EC-100 (N=33)	522 $\pm$ 45	1955 $\pm$ 203	nd	nd	3.75 $\pm$ 0.26	nd

<sup>a</sup>p<0.05, \*\*p<0.01, \*\*\*p<0.001, in comparison with respective control value  
 alitters were culled to 8 pups on the day of birth nd=not determined

**Table 3** Effect of exposure of male rats, throughout gestation and until day 22 postnatal, to diethylstilboestrol (DES), octylphenol (OP), octylphenol + 5 ethoxylate groups (OP-5EO) or butyl benzyl phthalate (BBP) added to drinking water ( $\mu\text{g/L}$ ), on bodyweight and the weights of the testis, kidney and ventral prostate (mean $\pm$ SD) at age 90-95 days (Study 2)

Treatment Group ( $\mu\text{g/L}$ ) <sup>a</sup>	Body weight(g)	Organ Weights (mg)			Relative organ weight (mg/g BW)			
		Testis	Kidney	Ventral prostate	Testis	Kidney	Ventral prostate	
Control (N=26)	489 $\pm$ 32	2014 $\pm$ 155	1837 $\pm$ 118	468 $\pm$ 79	1.10 $\pm$ 0.08	4.12 $\pm$ 0.26	3.76 $\pm$ 0.19	0.96 $\pm$ 0.14
DES-100 (N=26)	445 $\pm$ 41**	1750 $\pm$ 180***	1759 $\pm$ 158*	393 $\pm$ 44**	1.00 $\pm$ 0.09***	3.94 $\pm$ 0.34*	3.96 $\pm$ 0.24*	0.89 $\pm$ 0.11
OP-1000 (N=27)	530 $\pm$ 53***	1899 $\pm$ 123**	1915 $\pm$ 160*	428 $\pm$ 88	0.99 $\pm$ 0.07***	3.61 $\pm$ 0.32***	3.63 $\pm$ 0.32	0.82 $\pm$ 0.19**
OP-100 (N=29)	534 $\pm$ 41***	2042 $\pm$ 179	1880 $\pm$ 112	442 $\pm$ 82	1.09 $\pm$ 0.10	3.84 $\pm$ 0.38**	3.53 $\pm$ 0.23**	0.84 $\pm$ 0.18*
OP-5EO-1000 (N=32)	461 $\pm$ 32*	1783 $\pm$ 137***	1796 $\pm$ 159	476 $\pm$ 103	1.00 $\pm$ 0.11***	3.88 $\pm$ 0.30**	3.90 $\pm$ 0.35	1.03 $\pm$ 0.20
BBP-1000 (N=35)	476 $\pm$ 28	1809 $\pm$ 126***	1630 $\pm$ 116	454 $\pm$ 84	0.99 $\pm$ 0.09***	3.81 $\pm$ 0.34***	3.85 $\pm$ 0.24	0.96 $\pm$ 0.19

\* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , in comparison with respective control value

<sup>a</sup>Litters were culled to 8 pups on the day of birth

**Table 4** Effect of exposure of male rats, throughout gestation and until day 22 postnatal, to diethylstilboestrol (DES), octylphenol (OP), octylphenol + 5 ethoxyate groups (OP - 5EO) or butyl benzyl phthalate (BBP) added to drinking water ( $\mu\text{g/L}$ ), on bodyweight and the weights of the testis, kidney and ventral prostate (means  $\pm$  SD) at age 90-95 days (Study 3).

Treatment Group ( $\mu\text{g/L}$ )	Body weight(g)	Organ Weights (mg)			Relative organ weight (mg/g BW)			
		Testis	Kidney	Ventral prostate	Testis/kidney weight ratio	Testis	Kidney	Ventral prostate
Control (N=36)	479 $\pm$ 26	1954 $\pm$ 118	1749 $\pm$ 169	522 $\pm$ 84	1.13 $\pm$ 0.14	4.09 $\pm$ 0.28	3.66 $\pm$ 0.33	1.08 $\pm$ 0.16
DES-100 (N=23)	466 $\pm$ 34	1847 $\pm$ 157**	1792 $\pm$ 183	387 $\pm$ 83***	1.04 $\pm$ 0.16**	3.99 $\pm$ 0.45	3.85 $\pm$ 0.30*	0.84 $\pm$ 0.18***
OP-1000 (N=37)	474 $\pm$ 34	1696 $\pm$ 140***	1621 $\pm$ 121**	461 $\pm$ 63**	1.05 $\pm$ 0.07**	3.57 $\pm$ 0.23***	3.42 $\pm$ 0.24***	0.98 $\pm$ 0.11**
OP-100 (N=34)	461 $\pm$ 22*	1838 $\pm$ 114**	1693 $\pm$ 121	470 $\pm$ 76*	1.09 $\pm$ 0.09	4.00 $\pm$ 0.31	3.69 $\pm$ 0.22	1.03 $\pm$ 0.20
OP-5EO-1000 (N=34)	460 $\pm$ 32*	1810 $\pm$ 110***	1710 $\pm$ 160	495 $\pm$ 65	1.07 $\pm$ 0.11*	3.95 $\pm$ 0.35*	3.72 $\pm$ 0.22	1.08 $\pm$ 0.12
BBP-1000 (N=35)	477 $\pm$ 24	1819 $\pm$ 119***	1830 $\pm$ 133	486 $\pm$ 63	1.01 $\pm$ 0.08***	3.82 $\pm$ 0.26**	3.79 $\pm$ 0.20	1.03 $\pm$ 0.12

\*p<0.05, \*\*p<0.01, \*\*\*p<0.001, in comparison with respective control value

all litters NOT culled to a standard size at birth

b) Litter size etc also affected by treatment (see Table 1)

**Table 5** Quantitative analysis of the cross-sectional area of seminiferous tubules and the seminiferous epithelium at stages VII-VIII of the spermatogenic cycle in individual animals from the various treatment groups in Study 3. Data are the mean  $\pm$  SD for ten seminiferous tubules per animal and were based on analysis of perfusion-fixed tissue.

Treatment Group	Animal No.	Seminiferous tubule area ( $10^3 \mu\text{m}^2$ )	Seminiferous epithelium area ( $10^3 \mu\text{m}^2$ )
Control	1	77 $\pm$ 9	56 $\pm$ 6
	2	71 $\pm$ 9	51 $\pm$ 6
DES - 100 $\mu\text{g/L}$	1	94 $\pm$ 11	69 $\pm$ 6
	2	89 $\pm$ 12	68 $\pm$ 8
OP- 1000 $\mu\text{g/L}$	1	79 $\pm$ 7	58 $\pm$ 5
	2	74 $\pm$ 6	56 $\pm$ 5
	3	76 $\pm$ 9	61 $\pm$ 6
OP-5EO - 1000 $\mu\text{g/L}$	1	83 $\pm$ 9	61 $\pm$ 8
	2	86 $\pm$ 12	63 $\pm$ 8
BBP - 1000 $\mu\text{g/L}$	1	86 $\pm$ 11	62 $\pm$ 8
	2	86 $\pm$ 9	64 $\pm$ 7

Because of the small numbers of animals, no statistical analysis of the above data was attempted

**SCHENECTADY INTERNATIONAL, INC.**

**SUBJECT:**      Effects of Trace Organics  
                 on Fish--Phase 2

**DATE:**      August 15, 1995

**TO:**            APE Panel

**FROM:**      Robert P. Yunick

During the July 26 press flurry in the U.K., mention was made in The Times of a so-called "Brunel Study" which had been released on July 25 along with the IEH report. R6

I received a copy of the report today (copy of the title page, contents and summary attached) and found that it is the previously referred to "MAFF Report" from which Peter Matthiessen shared some information with us at the EPA Estrogen Seminar in April at RTP, NC.

Please note that the full report, 90 pp., is available for £25 from the Foundation for Water Research--full details on the enclosed title page. Based on a very quick perusal of the report, it appears to be mandatory reading.

AP compounds are the subject of Chapter 4, pp42-51; while all other estrogenic compounds (19 listed) occupy pp. 62-70 of Chapter 5.

In the recommendations in Chapter 7, the first reads, in part, "At present there is insufficient evidence to justify general regulatory action, other than further research into the causes of these effects and possible implications for fish populations." It proceeds to recommend further fresh- and salt-water (estuaries) species testing, and additional bioassay testing of sewage effluent fractions to identify all "major environmental oestrogens." These MEO's should then be tested in vivo in fish.

Nandy--please share this with Elizabeth Watson.

RPY/cbd

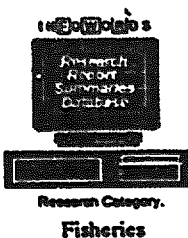
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**EFFECTS OF TRACE ORGANICS ON FISH  
PHASE 2**

**Contractors:  
MAFF Fisheries Research Directorate;  
Department of Biology & Biochemistry,  
Brunel University**

**July 1995**

**FR/D 0022**



## **EFFECTS OF TRACE ORGANICS ON FISH - PHASE 2**

**FR/D 0022**

**JULY 1995**

**PREPARED FOR  
THE DEPARTMENT OF  
THE ENVIRONMENT**

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the Department of the Environment by**

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# CONTENTS

<b>Executive Summary .....</b>	<b>1</b>
<b>1. Introduction.....</b>	<b>5</b>
<b>2. Survey of raw water storage reservoirs in the southeast of England.....</b>	<b>8</b>
<b>3. Surveys in 6 English rivers for oestrogenic activity</b>	
<b>3.1. River Lea: July-August 1992 .....</b>	<b>15</b>
<b>3.2. River Lea: November-December 1992.....</b>	<b>23</b>
<b>3.3. Dilution studies at Harpenden STW and in the River Lea 1994 .....</b>	<b>29</b>
<b>3.4. 4 rivers in Southern England, 1994.....</b>	<b>34</b>
<b>3.5. River Aire, West Yorkshire 1994 .....</b>	<b>38</b>
<b>4. Oestrogenicity of alkylphenolic compounds .....</b>	<b>42</b>
<b>5. A search for other oestrogenic chemicals.....</b>	<b>62</b>
<b>6. Discussion and conclusions.....</b>	<b>71</b>
<b>7. Recommendations .....</b>	<b>79</b>
<b>8. References .....</b>	<b>81</b>
<b>9. Appendices</b>	
<b>Appendix 2.1 Sensitising the Radioimmunoassay .....</b>	<b>86</b>
<b>Appendix 2.2 Formulae for somatic and condition indices .....</b>	<b>87</b>
<b>Appendix 2.3 Physical data for reservoir trials: July-August 1993 .....</b>	<b>88</b>
<b>Appendix 3.1 Physical Data - R Lea: July-August 1992 .....</b>	<b>89</b>
<b>Appendix 4.0 List of raw data not appended in this report</b>	
<b>(Available from DOE on request) .....</b>	<b>90</b>

## EXECUTIVE SUMMARY

1. The work reported here follows on from an earlier DoE-funded study which had shown that treated sewage effluent discharges were oestrogenic to fish. The report describes research conducted in 1992-1995 to assess whether oestrogenic effects could be detected in male fish held in cages at selected sites in rivers below sewage treatment works, and at downstream points either where raw water is abstracted for treatment before public supply, or in raw water storage reservoirs<sup>1</sup>. The basic survey technique involved holding caged male rainbow trout in the river or raw water storage reservoir for 3-6 weeks and measuring production of a protein (vitellogenin or VTG) which is a specific marker for the presence of oestrogens. Production of vitellogenin is confined to egg-laying vertebrates and is described as an oestrogenic effect because in female egg-laying vertebrates it is usually controlled by hormones known as oestrogens. The research also set out to determine possible causes of the observed oestrogenic responses; it seemed probable that some substances in sewage effluents could be acting as oestrogens in the male fish.

2. Of the 15 raw water storage reservoirs<sup>(1)</sup> that were surveyed, it was shown that none produced a vitellogenin response in caged fish. On the basis that biologically significant amounts of substances, which are responsible for the oestrogenic response (in fish), were not detected in all 15 raw water storage reservoirs most likely to be affected, there is no evidence of a risk to drinking water supplies, and for the present there is no need for further research.

3. The research covered stretches of six rivers: the Sussex Arun, the Kent Stour, the Hertfordshire Lea, the Essex Chelmer, the Suffolk Stour and the West Yorkshire Aire. In five of the rivers, work focused on a single stretch below a particular sewage treatment works (STW) outfall, while in the Lea surveys were made downstream of several STW discharges. The Aire was chosen because it is known to contain alkyl phenol ethoxylates from wool scouring; it is not used for abstraction of raw water<sup>(2)</sup>. In all, 10 river stretches were surveyed.

<sup>1</sup>Raw water storage reservoirs are reservoirs from which water is drawn for extensive physical, chemical and sometimes biological treatment at a treatment works before being supplied as drinking water.

<sup>2</sup>Raw water undergoes extensive treatment at a water treatment works before being supplied as drinking water.

4. For four of the ten surveyed stretches (in the Lea, the Kent Stour and the Chelmer), the oestrogenic response in caged fish was confined to the undiluted sewage effluent discharges and was not observed downstream. For five of the ten river stretches, in the Arun, Lea and Aire, the response was observed close to the sewage effluent discharges and downstream; on the Arun the response was observed up to 1.5 km downstream of the discharge and was not detected further downstream; on the Lea and the Aire the response was detected up to 5 km below the particular discharges, although the response in the Lea declined rapidly with distance downstream. The magnitude of the response in these five river stretches varied widely, from relatively small responses in the Arun through moderate responses in the Lea, to large responses in the Aire. For the remaining surveyed stretch on the river Stour (Suffolk) neither the sewage effluent discharge nor the river downstream were oestrogenic to caged fish.

5. Of the 4 sites that were surveyed close to points on the rivers Kent Stour, Chelmer, Essex Stour and Lea where raw water is abstracted for treatment prior to entering public supply, it was shown that none produced a vitellogenin response in caged fish.

6. In the case of the Aire, heavy stimulation of vitellogenin synthesis in caged male trout to levels similar to those in gravid females occurred at all sites tested in summer 1994 over a 5 km stretch downstream of Marley STW. This synthesis of vitellogenin was accompanied by reduced testicular development. A similar effect on the testes was seen in fish deployed at seven sites on the Lea during summer 1992. These observations show that exposure to oestrogenic substances can have effects on fish with potential implications for their ability to breed normally. However, the implications of these short term observations of reduced testicular growth for fish reproduction and populations are not yet known.

7. Dilution experiments at a STW on the Lea which had strongly oestrogenic effluent showed that 2 or more times dilution of unfiltered effluent was sufficient to prevent the vitellogenin response in fish. Because STWs are often sited along river stretches where the effluent is diluted at least 2 times, the results from the dilution study could suggest that the majority of STW discharges are unlikely to cause oestrogenic effects in fish held in their receiving waters. However, additional dilution experiments with different types of discharge would be required to confirm this point. These experiments also suggest that oestrogenic

effects are likely to be greater in summer when dilution is less than in winter. Indeed, the smaller responses seen in the River Lea in winter support this prediction, although other influences such as temperature could have contributed to these effects.

8. The likely ecological effects of oestrogenic river water have not yet been established, although it is clear that oestrogens have the potential to disrupt reproduction in vertebrates such as fish, perhaps leading to long term declines in population size. Limited evidence presented in this report shows that some free-living wild fish (roach) are responding to oestrogenic substances in the environment so the possibility exists of localised ecological impacts.

9. Whilst the identity of the oestrogens in sewage effluent, responsible for these effects, is still largely unknown, this project provided useful data about the oestrogenicity of a number of substances known to be present in sewage effluent. Alkylphenol ethoxylates, which are used as surfactants in industrial detergents, and their degradation products, were shown to be weakly oestrogenic to fish in laboratory experiments (in vitro and in vivo), possessing around one ten-thousandth the potency of the natural oestrogen, 17 $\beta$ -oestradiol. Of the 20 chemicals selected on the basis of their likely occurrence in sewage effluent and examined for oestrogenic activity using a number of screening methods, 9 were shown to be weakly oestrogenic. This further supports the hypothesis that a number of substances in sewage effluent could be acting as oestrogens in the male fish in this project.

10. The hypothesis that ethinyl estradiol (EE2), the synthetic oestrogen present in the contraceptive pill, is partly responsible for our observations was not resolved by this project, although women taking oral contraceptives excrete the conjugated form of EE2 which is not oestrogenically active. Some preliminary laboratory work (MAFF unpublished data) has shown that this inactive form of EE2 may be activated by enzymes present in sewage. Brief and unsuccessful attempts were made in this project to detect EE2 in sewage effluent using a sensitive radioimmunoassay, but the technique was found to be of limited success because of interferences present in the sewage effluent.

11. On the basis of the laboratory work and residue measurements in the field, it seems likely that the majority of the effect seen in the surveyed stretch of the River Aire can be

explained by the presence of high levels of a degradation product of nonylphenol ethoxylates. Alkylphenols and their parent ethoxylates may also be responsible for the effects seen in the other river surveyed because this group of chemicals is in wide use in industrial detergents and is known to be present in rivers. However, it is likely that a wide range of unrelated substances is contributing to the effects, and that different substances will dominate in different rivers according to local inputs. Concentrations of nonylphenol and alkylphenol ethoxylates vary (within and) between rivers but are generally much lower than levels seen on the Aire.

12. The report concludes with a series of recommendations for further research to answer more comprehensively the question of which substances are responsible for the effects, which habitats are the most affected, what the ecological implications may be.

Table 1. Uterotropic activities of diethylstilbestrol (DES) and octylphenol (OP) in immature female rats

Group	n	Body weight (g)	Uterine wt (mg)
Vehicle	5	56 ± 2	39 ± 4
DES	5	48 ± 1*	141 ± 12**
OP	5	58 ± 1	78 ± 6**

\* $P < 0.05$ , \*\* $P < 0.01$  vs vehicle group (Wilcoxon signed-rank test).

## MATERIALS AND METHODS

### Experiment 1

Three groups of 23-day-old female Wistar rats received s.c. injections on 3 consecutive days of 100  $\mu$ l ethyl oleate vehicle, alone or containing either 5  $\mu$ g of DES (Sigma, Poole, U.K.) or 10 mg of OP (2000-fold higher mass dose than DES; 4-(*tert*-octyl) phenol; Aldrich, Gillingham, U.K.). 24 hours following the last injection, rats were sacrificed, their uteri excised and weighed and fixed in formaldehyde.

### Experiment 2

On each of the last 4 days of gestation, pregnant female rats received s.c. injections of 100  $\mu$ l ethyl oleate vehicle, alone or containing either 20  $\mu$ g DES or 40 mg OP (2000  $\times$  DES dose). Following delivery of litters, all pups received a daily s.c. injection on postnatal days 1-4 of 50  $\mu$ l ethyl oleate alone or containing either 1  $\mu$ g

DES or 2 mg OP (2000  $\times$  DES dose). Litters were weaned at 21 days and sexes caged separately from 24 days of age. At 60 days of age, a maximum of 6 male and female rats per treatment were deeply anaesthetized with Avertin and transcardially perfused with heparinized saline followed by 10% formaldehyde in phosphate buffered saline. Whole brains were removed and immersion fixed for 10 days prior to embedding, sectioned at 60  $\mu$ m through the preoptic area and stained with thionin. Coded slides were examined blind and the boundaries of the SDN-POA on each side of the brain drawn using a camera lucida. SDN-POA area was calculated using computer-based image analysis. Testes were dissected and weighed following removal of the epididymis.

## RESULTS

DES induced a 260% increase in uterine weight ( $P < 0.01$ ) accompanied by a 15% decrease in body weight. OP was active in inducing a 100% increase in uterine weight ( $P < 0.01$ ) without a change in body weight (Table 1 and Fig. 1).

In confirmation of earlier reports, the SDN-POA area was 115% greater ( $P < 0.01$ ) in vehicle treated males than in females; and DES significantly increased SDN-POA area in females by 46% (Table 2). OP treatment was found to have no effect on SDN-POA morphology. Neither compound influenced SDN-POA area in males. Body weight was slightly increased in females by both DES and OP perinatally. In males,

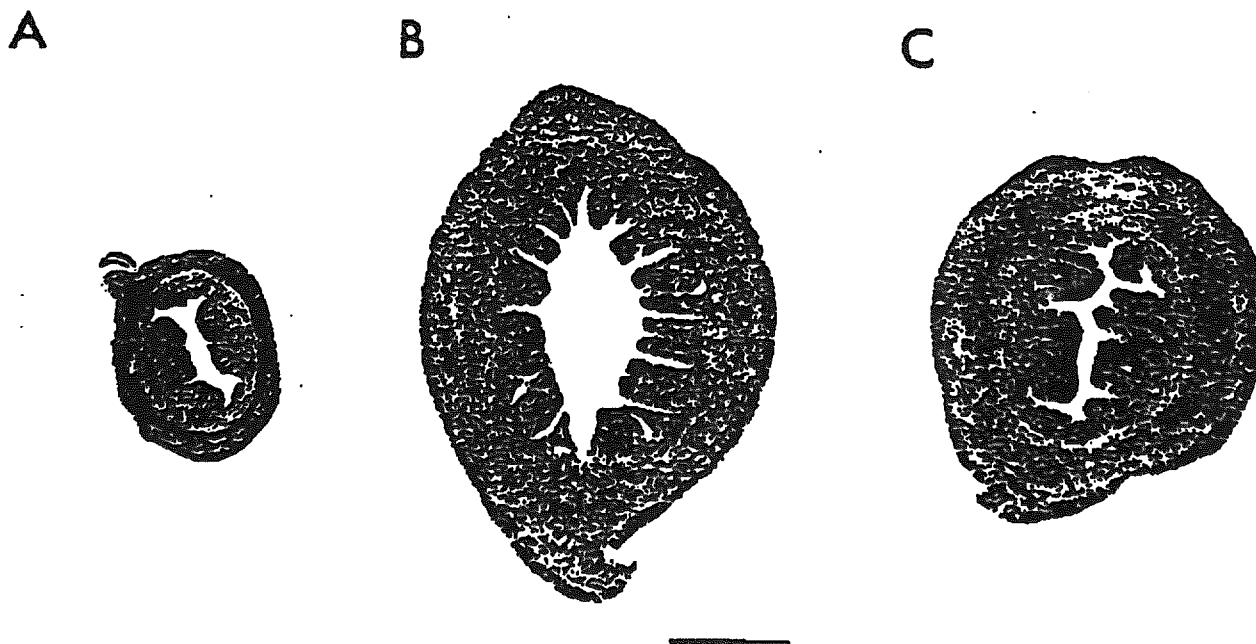


Fig. 1. Histological appearance of rat uterus following treatment with A, vehicle; B, DES and C, OP. Transverse sections stained with haematoxylin and eosin. Scale bar, 0.5 mm.

# Oestrogenic Activity of an Alkylphenol

9

Table 2. Effects of perinatal exposure to DES and OP on area of the sexually dimorphic nucleus of the preoptic area (SDN-POA) of the brain of rats 60 days postnatally

Females				Males		
Group	n	Body weight (g)	Area of SDN-POA (mm <sup>2</sup> )	n	Body weight (g)	Testis weight/100 g body weight (g)
Vehicle	6	183 ± 4	0.0325 ± 0.0026	6	298 ± 9	0.716 ± 0.068
DES	6	196 ± 3*	0.0475 ± 0.0039*	3	267 ± 18	0.635 ± 0.142
OP	6	200 ± 3*	0.0362 ± 0.0020	6	314 ± 9	0.874 ± 0.058
						0.0590 ± 0.0032

\*P < 0.05, \*\*P < 0.01 vs vehicle female group (Wilcoxon signed rank test).

combined testis weight was 30% greater following OP but this difference was not significant when expressed per unit body weight (Table 2).

## DISCUSSION

Alkylphenols such as OP have recently been described to bind directly to oestrogen receptors from trout, to stimulate vitellogenin gene expression in trout hepatocytes, to be mitogenic in human breast cancer cell lines and to stimulate transcription in mammalian and avian cell cultures through the oestrogen receptor [7].

To our knowledge this is the first report on the potential *in vivo* oestrogenicity of alkylphenols in a mammalian species. These early findings lead us to conclude that OP is weakly oestrogenic at peripheral target tissues when administered subcutaneously, but that acute perinatal exposure does not reduce gross testis development. The lack of effect of OP on brain sexual differentiation may be due to insufficient penetration into the brain, to the relative lack of sensitivity of this process or to intrinsic inactivity of OP at neural oestrogen receptors. In contrast, the masculinization of the female SDN-POA by DES shown here is in good agreement with previous reports [9]. Although the nature of gonadal steroid action on the SDN-POA appears relatively well defined, its physiological significance with respect to sexually differentiated neural functioning remains unclear [9]. On a mass basis, OP is more than 2000 times less effective than DES in our study and some 1000-fold less active than 17 $\beta$ -oestradiol *in vitro* [7]. Further information on dose, duration and routes of exposure to alkylphenols will be needed to assess the impact of these degradation products of

a widely used group of surfactants on health and reproduction in human and wildlife populations.

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## Rapid Communication

# Oestrogenic Activity of an Environmentally Persistent Alkylphenol in the Reproductive Tract but not the Brain of Rodents

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Compounds with oestrogenic actions present in the environment as a result of human activity may represent a threat to health and reproductive efficiency in human and wildlife populations. We show here that parenteral administration of octylphenol, a recently described environmental oestrogen derived from one group of non-ionic surfactants, is active in stimulating oestrogen-dependent uterine growth in prepubertal rats, but has no influence on perinatal sexual differentiation of the rat brain. These results extend previous *in vitro* findings to show that alkylphenols exert weak oestrogenic activity *in vivo* in mammals.

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## INTRODUCTION

An increasing concern in human reproductive medicine is the possible adverse effect of xenobiotic oestrogens in the environment or diet. Thus, attention has been drawn to the possible role of exposure to environmental oestrogens in abnormal gonad development, decreased sperm quality and oestrogen-sensitive breast cancer in humans [1-3] and wildlife populations are also believed to be at risk from environmental oestrogenic pollution [4]. A new class of xenobiotic oestrogens, alkylphenolic compounds, derived from degradation of one class of non-ionic surfactants added to detergents, toiletries, herbicides etc (annual global production over 300,000 tons), have recently been described in sewage effluent and are oestrogenic in fish [5]. Alkylphenols such as nonyl- or octylphenol are also oestrogenic in mammalian breast cancer cell proliferation assays [6] and are transcriptionally active *in vitro* through the oestrogen receptor [7].

Since it is important to know the bioavailability and oestrogenicity of this class of compound in the mam-

malian body to assess their possible impact, we have carried out two preliminary experiments delivering octylphenol (OP) to rats. Firstly, we examined the ability of OP to stimulate uterine growth in prepubertal female rats, a well established oestrogenic bioassay [8]. Specific areas of the rodent and human brain exhibit morphological sex differences [9, 10]. The sexually dimorphic nucleus of the preoptic area (SDN-POA) in the rat is the best characterized of these regions and sex differences in its cytoarchitecture are known to be critically dependent on perinatal oestrogen exposure [9]. Aromatization of testosterone to oestrogen in the brain of the male ensures high local concentrations of oestrogen resulting in an SDN-POA of substantially larger volume and cross sectional area. An SDN-POA of male proportions can similarly be induced in female rats by the perinatal administration of synthetic oestrogens such as diethylstilbestrol (DES) [9]. Thus, in a second experiment we have examined the ability of OP to induce oestrogen-dependent sex differences in the SDN-POA of the rat and compared its actions with DES using the same protocol of perinatal steroid administration as these earlier studies [9] and relative doses based on oestrogenic potency of OP *in vitro* [7].

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**Table 6** Estimation of the nominal intake of octylphenol (OP) and butyl benzyl phthalate (BBP) between birth and day 21 of postnatal life, based on water consumption in Study 3 (Means  $\pm$  SD, N=6)

Treatment Group	Days postnatal	Water intake (ml/48 h)	Nominal mean intake of chemical ( $\mu$ g/kg/day) <sup>a</sup>
Control	1+2	75 $\pm$ 22	-
	10+11	182 $\pm$ 55	-
	20+21	243 $\pm$ 72	-
OP - 1000 $\mu$ g/L	1+2	90 $\pm$ 36	129
	10+11	216 $\pm$ 42	309
	20+21	257 $\pm$ 69	367
BBP-1000 $\mu$ g/L	1+2	88 $\pm$ 24	126
	10+11	192 $\pm$ 64	274
	20+21	256 $\pm$ 43	366

<sup>a</sup> Assumes a bodyweight of 350 g in the lactating female

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